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Communications

Adducts of Acrylonitrile with Hemoglobin in Nonsmokers and in Participants in a Smoking Cessation Program

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Hemoglobin adducts have been used to assess exposure to carcinogenic compounds in tobacco smoke. However, because of background levels in nonsmokers, most adducts that have been studied are not useful for monitoring low-level exposure. Bergmark [(1997) *Chem. Res. Toxicol. 10*, 78−84] showed that the level of adducts of acrylonitrile (AN) with N-terminal valine (ANVal) increases with increasing cigarette consumption, and the increment from 1 cigarette/day was estimated to be 8 pmol/g of globin. The background level of ANVal in nonsmokers was not quantified (<2 pmol/g of globin). The objective of this study was to determine the background level of ANVal in hemoglobin and to study the stability of this adduct in vivo. Globin samples previously analyzed by Bergmark from 17 nonsmokers and 2 smokers were reanalyzed in the study presented here. Globin samples from 7 additional nonsmokers and from 10 participants in a smoking cessation program were also analyzed. Smoking habits and exposure to environmental tobacco smoke (ETS) were assessed by interview. Only two of the participants completed the program. The levels of ANVal in these 2 subjects decreased after quitting and were at background level by 126 days. The time course of the decrease was compatible with removal of stable adducts. The levels of ANVal in the nonsmokers were 0.76 ± 0.36 (mean ± SD) (n = 18; reporting no exposure ETS), 1.1 ± 0.6 (n = 3; reporting exposure to ETS), and 1.2 ± 0.5 pmol/g of globin (n = 3; snuff users). Thus, the adduct level in nonsmokers corresponds to the adduct increment from about 0.1 cigarette/day. Measurements of the level of ANVal could be used to distinguish between nonsmokers and low-level smokers on an individual level, but larger groups of individuals would be required to detect a possible contribution to the background from passive smoking.

Introduction

Tobacco smoke contains a large number of compounds, including several well-known irritants and carcinogens. As an aid in epidemiological studies of the adverse health effects of tobacco smoke, it is desirable to supplement existing standard methods for assessment of exposure, such as measurements of cotinine concentration and interviews, with tests that provide more reliable information about the extent of exposure to genotoxic compounds

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Table 1. Background Level of Various Hemoglobin Adducts in Nonsmokers and the Adduct Increment from 1 Cigarette/Day[a,b]

reactive component of tobacco smoke	adduct level *A* in nonsmokers (pmol/g of globin)	increment per cigarette per day *B* (pmol/g of globin)	*B*/*A* ratio
ethylene or ethylene oxide	20	8	0.4
methylating compounds	220	3.5	0.016
4-aminobiphenyl	0.13	0.03	0.2
acrylamide	31	6	0.2
acrylonitrile[c]	1	8	8

[a] References are given in the text. [b] Adducts with cysteine for 4-aminobiphenyl and adducts with N-terminal valine for the other compounds. [c] This study.

in tobacco smoke. Interview answers are to some extent subjective, and cotinine concentrations give only information about the last day's exposure.

Measurement of the levels of stable reaction products (adducts) of genotoxic agents with hemoglobin gives a measure of in vivo doses obtained during the 3−4 months preceding blood sampling (*1*, *2*). One of the prerequisites for using a particular adduct as a monitor of exposure to tobacco smoke is that the smoke be the dominating cause of the adduct formation. Many of the adducts previously studied are also found in nonsmokers, compromising their use for monitoring low-level exposure to tobacco smoke. Another problem in the comparison between smokers and nonsmokers is the different distribution of different genotoxic compounds among side stream smoke (SS)[1] and main stream smoke (MS). Further, in environmental tobacco smoke (ETS), SS and exhaled MS have subsequently undergone modification due to deposition and aging.

The background levels of various hemoglobin adducts in nonsmokers and the adduct increment from 1 cigarette/day are compared in Table 1. Adducts of ethylene or ethylene oxide in nonsmokers mainly originate from endogenously produced ethylene, and the background level corresponds to a few cigarettes per day (*3*, *4*). Adducts of methylating agents exhibit a high background of endogenous origin that excludes measurement of methylation as an index of smoking (*4*). Another adduct present at increased levels in smokers is formed from acrylamide. However, the background level of this adduct is relatively high compared to the increment caused by smoking (*5*).

Bryant et al. (*6*) demonstrated a correlation between the number of cigarettes per day and the level of adducts of 4-aminobiphenyl (4-ABP) with cysteine in hemoglobin. The main source of the background of these adducts is believed to be ETS, although dietary contamination and ambient concentrations of 4-ABP may contribute (*7*). Hammond et al. (*8*) demonstrated a correlation between exposure to nicotine in the air and the level of 4-ABP adducts in nonsmoking women. The low ratio between the adduct increment from 1 cigarette/day and the background adduct level (*B*/*A* in Table 1) may be related to the 30-fold higher content of 4-ABP in SS than in MS.

Adducts from the tobacco specific nitrosoamines NNN and NNK are identified after hydrolysis of hemoglobin. The highest levels have been analyzed in snuff users [0.517 ± 0.538 pmol/g of hemoglobin (mean ± SD)]. The values recorded for cigarette smokers (0.0796 ± 0.189 pmol/g) and nonsmokers (0.0293 ± 0.0259 pmol/g) overlap (*9*). The difference between snuff users and cigarette smokers and the overlap of values for smokers and nonsmokers cannot easily be explained by differences in uptake of NNN and NNK. Interindividual differences in metabolism or possibly in endogenous production of NNN and NNK from precursors may play a role.

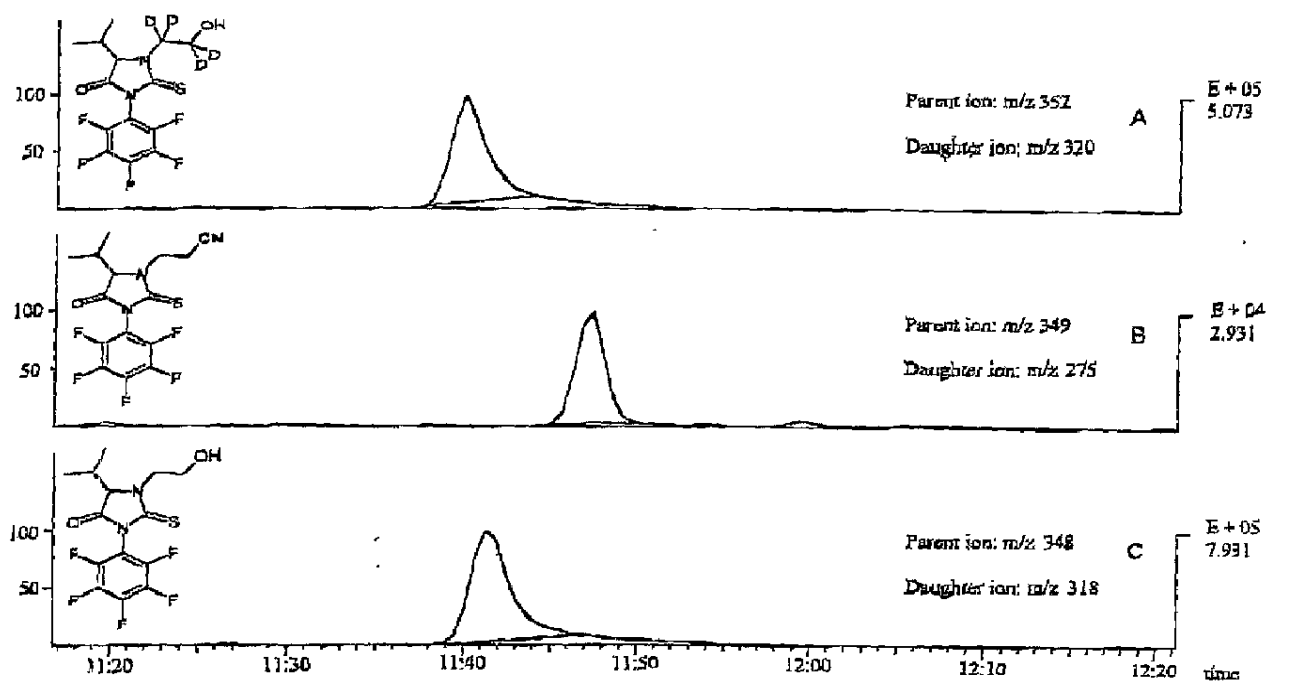

Figure 1. Ion chromatogram from GC/MS−MS analysis of globin from a nonsmoker: (A) internal standard ($^2H_4$)HOEtVal−PFPTH, (B) ANVal−PFPTH, and (C) HOEtVal−PFPTH.

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Apparently, a set of adducts has to be chosen to give a pertinent description of exposure to the carcinogens in tobacco and tobacco smoke. The objective of this study was to determine the background level of adducts of acrylonitrile (AN), a component of tobacco smoke, with N-terminal valine in hemoglobin and to study the stability of these adducts in vivo.

AN is an important industrial chemical which is used extensively in the manufacture of synthetic fibers, resins, plastics, and rubber for a variety of consumer goods. Humans may be exposed to AN in the workplace, through contaminated drinking water (*10*) and via tobacco smoke (*11*). Approximately 3.9 times as much AN is emitted in SS as in MS (39 vs 10 μg/cigarette).

AN reacts with the N-terminal valine in hemoglobin, forming the adduct *N*-(2-cyanoethyl)valine (ANVal) (*2*, *12*):

$$\text{globin}-\text{Val-NH}_2 + \text{CH}_2{=}\text{CHCN} \rightarrow \text{globin}-\text{Val-NH-CH}_2\text{CH}_2\text{CN}$$

Bergmark (*5*) showed a linear relationship between adduct level and tobacco consumption (about 8 pmol of ANVal per gram of globin per cigarette per day). The adduct levels in 23 nonsmokers were all below the detection level (<2 pmol/g of globin). Tavares et al. (*13*) measured ANVal levels in smoking and nonsmoking mothers and in their newborns. There was a correlation between the adduct levels in both the mothers and the newborns and the number of cigarettes per day smoked by the mother. The adduct level in nonsmokers was below the detection limit of the assay (1 pmol/g of globin).

Study Design

Blood samples were collected from 10 participants in a smoking cessation program who gave blood 1 day, 4–8 days, 28 days, and 4 months after quitting and from 7 nonsmokers. In addition, globin samples from 17 nonsmokers and 2 smokers previously analyzed by Bergmark (*5*) were reanalyzed in this study. The subjects filled out a questionnaire form about their smoking habits, snuff intake, and exposure to ETS.

ANVal was quantified using the modified Edman method with detection by GC/MS–MS. For details, see Bergmark (*5*). A globin, containing 2.5 pmol/mg of ($^2H_4$)hydroxyethylvaline ($[^2H_4]$HOEtVal), was used as internal standard. Globin samples (100–200 mg), dissolved in 6 mL of formamide, 160 μL of 1 M sodium hydroxide, and 2 μg of internal standard globin, were incubated with 40 μL of pentafluorophenyl isothiocyanate overnight at room temperature to prepare the pentafluorophenylthiohydantoin (PFPTH) derivatives. The reaction was completed at 45 °C for 1.5 h. The PFPTH derivatives were extracted with 3 × 3 mL of diethyl ether. The ether extracts were evaporated under nitrogen, and the sample was dissolved in 2 mL of toluene. The toluene phase was washed twice with 1 mL of 0.1 M sodium carbonate and twice with 1 mL of water. Toluene was evaporated, and the samples were reconstituted in 50 μL of toluene for GC/MS–MS analysis. The PFPTH derivatives were analyzed in the negative ion chemical ionization mode. Figure 1 shows the ion chromatogram from the GC/MS–MS analysis of globin from one of the nonsmokers. The parent and daughter ions that were selected for monitoring are presented in the figure.

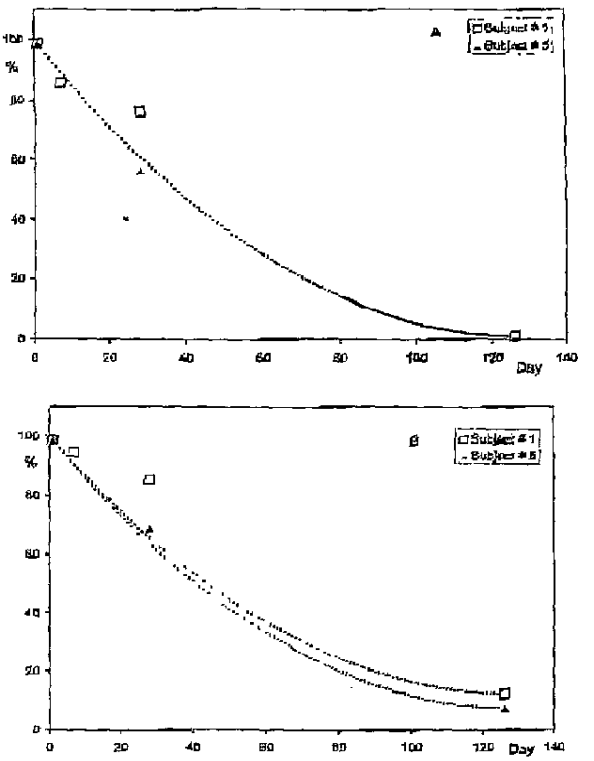

Figure 2. Levels (normalized) of hemoglobin adducts over time in 2 individuals who completed a smoking cessation program. The theoretical elimination curves (dotted lines) were generated for each individual using eq 1, were the adduct level at day 0 was set to 100% and the adduct level at 126 days (the erythrocyte life span) was used as a background: (A) ANVal and (B) HOEtVal. The initial levels were 54 pmol of ANVal/g and 73 pmol of HOEtVal/g for subject 1 and 91 pmol of ANVal/g and 87 pmol of HOEtVal/g for subject 5.

A tripeptide, *N*-(2-cyanoethyl)[^{14}C]valylglycylglycine ethyl ester (0–40 pmol) prepared essentially as described by Lissa Pérez et al. (*14*), and 2 μg of internal standard [5 pmol of ($^2H_4$)HOEtVal] were used for calibration. The samples were processed as described above. The calibration curve was linear in the concentration range that was studied ($R^2 = 0.98$).

The improved sensitivity of the assay, as compared to that in the previous study by Bergmark (*5*), was mainly achieved by using larger amounts of globin (100–200 vs 50 mg) and by extending the calibration curve down to 0.05 pmol (0.25 pmol/g of globin) of tripeptide in the sample. Values for HOEtVal, the adduct of ethylene oxide, were also recorded assuming a linear relationship between analyte and internal standard. Several earlier studies on this adduct demonstrate linear calibration curves down to the background (around 20 pmol/g of globin; see, for example, ref *3*).

Results and Discussion

Prolonged exposure to reactive chemicals results in steady state levels of hemoglobin adducts. Chemically stable adducts reach a level, which is about 63-fold (t_{av}/2, t_{av} being the life span of the erythrocytes) higher than the daily adduct increment (*a*). The less stable the adducts, the lower the steady state level as compared to *a* and the shorter the period of time covered by the adduct measurement (*15*).

[1]Abbreviations: 4-ABP, 4-aminobiphenyl; AN, acrylonitrile; ANVal, *N*-(2-cyanoethyl)valine; ETS, environmental tobacco smoke; GC/MS–MS, tandem mass spectrometry; HOEtVal, *N*-(2-hydroxyethyl)valine; MS, main stream smoke; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine; SS, side stream smoke; PFPITC, pentafluorophenyl isothiocyanate; PFPTH, pentafluorophenylthiohydantoin.

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Table 2. Acrylonitrile–Hemoglobin Adduct [N-(2-Cyanoethyl)valine] Levels in Nonsmokers, Snuffers, and Smokers

description of exposure	no. of subjects	adduct level (pmol/g of globin) [mean ± SD (range)]
nonsmokers		
reporting no exposure to ETS	18	0.76 ± 0.36 (0.32–1.6)
reporting passive smoking	3	1.1 ± 0.6 (0.6–1.7)
ex-smokers (4 months after cessation)	2	0.7 (0.6; 0.7)
snuff users	3	1.2 ± 0.5 (0.7–1.7)
smokers		
20 cigarettes/day (before cessation of smoking)	1	91
10–15 cigarettes/day (before cessation of smoking)	1	64
"partysmokers"[a]	3	8.6 (2.2–14.6)
1–20 cigarettes/day[c]	14	86.2 (8.3–178)
"partysmoker"[b]	1	8.9[d]
1 cigarette/day[c]	1	8.7[e]

[a] Data from Bergmark (5). [b] One of the 3 "partysmokers" (5). [c] One of the 14 smokers (5). [d] A value of 9.1 pmol/g of globin according to Bergmark (5). [e] A value of 8.3 pmol/g of globin according to Bergmark (5).

The removal of stable adducts on cessation of chronic exposure may be described by eq 1 (see refs 15 and 16):

$$A = A_{background} + at_{ac}/2 - at + (at^2/2t_{ac}) \quad t \le t_{ac} \quad (1)$$

where t is the time after the end of exposure, A is the adduct level at time t, and $A_{background}$ is a background.

The adduct levels at the beginning of the smoking cessation program are considered to represent steady state levels. The decline with time of chemically stable adducts would follow eq 1 in individuals who give up smoking. According to the interviews, only 2 of the 10 participants completed the program. Levels of ANVal and HOEtVal recorded in these two ex-smokers are shown in panels A and B of Figure 2. The levels were at a normal background ($A_{background}$) by 126 days. The time course of the decrease was compatible with eq 1. However, because of the small number of samples, a somewhat reduced stability of the adducts is not excluded. The ANVal levels in the participants who gave blood 4, 7, or 8 days after cessation of smoking were 94 ($n = 1$), 86 ± 2 ($n = 5$), and 80% ($n = 1$) of the initial level as compared to the expected values of 95.3, 90.7, and 89.2%, respectively. Previous studies have indicated that HOEtVal in hemoglobin of accidentally exposed workers is chemically stable (17). The parallel decline with time of the levels of ANVal and HOEtVal in the 2 ex-smokers suggests that ANVal is also chemically stable. Following the initial decrease, the adduct levels in participants who, according to the questionnaires, resumed smoking, increased again.

ANVal levels in the 26 nonsmokers who were investigated are presented in Table 2. For comparison, adduct levels in the 2 ex-smokers before they gave up smoking and the adduct levels reported by Bergmark (5) in 14 smokers are also shown. Globin samples from 2 of the 14 smokers were reanalyzed and showed virtually the same adduct levels as previously determined. This study shows that a low level of ANVal is present in nonsmokers. The background [0.76 ± 0.36 pmol/g of globin (mean ± SD)] corresponds to a cigarette consumption of approximately 0.1 cigarette/day and may originate from ETS and from other sources of AN. Therefore, measurements of ANVal levels could be used to distinguish between nonsmokers and low-level smokers. Larger groups of individuals would be required to establish a possible contribution to the background from passive smoking. In the snuff users, the level of ANVal (1.2 ± 0.5 pmol/g of globin) was not significantly increased above the background and the level of HOEtVal was within the normal range for nonsmokers. These findings were expected, since the increased levels of ANVal and HOEtVal in smokers originate from volatile compounds (AN and ethylene or ethylene oxide, respectively) in tobacco smoke.

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